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RESEARCH PAPER

Penta-O-galloyl-β-D-glucose ameliorates inflammation by inhibiting MyD88/NF-κB and MyD88/MAPK signalling pathways

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BACKGROUND AND PURPOSE

The gallnut of *Rhus chinensis* MILL and its main constituent penta-O-galloyl-β-D-glucose (PGG) inhibited NF-κB activation in LPS-stimulated peritoneal and colonic macrophages. Here we have investigated PGG mechanisms underlying anti-inflammatory effects of PGG *in vitro* and *in vivo*.

EXPERIMENTAL APPROACH

Male C57BL/6 mice (18–22 g, 6 weeks old) were used to prepare peritoneal and colonic macrophages and for the induction of colitis by intrarectal administration of 2,3,4-trinitrobenzene sulphonic acid (TNBS). A range of inflammatory markers and transcription factors were evaluated by ELISA, immunoblotting, flow cytometry and confocal microscopy.

KEY RESULTS

Expression of Toll-like receptor (TLR)-4 or Lipopolysaccharide (LPS) binding to TLR-4 in LPS-stimulated peritoneal macrophages was not affected by PGG. However PGG inhibited binding of an anti-MyD88 antibody to peritoneal macrophages, but did not reduce binding of anti–IL-1 receptor-associated kinase (IRAK1) and IRAK4 antibodies to the macrophages with or without transfection with MyD88 siRNA. PGG potently reduced the activation of IRAK1, NF-κB, and MAPKs in LPS- or pepetidoglycan-stimulated peritoneal and colonic macrophages. PGG suppressed IL-1β, TNF-α and IL-6 in LPS-stimulated peritoneal macrophages, while increasing expression of the anti-inflammatorycytokine IL-10. Oral administration of PGG inhibited colon shortening and myeloperoxidase activity in mice with TNBS-induced colitis, along with reducing NF-κB activation and IL-1β, TNF-α, and IL-6 levels, whereas it increased IL-10.

CONCLUSIONS AND IMPLICATIONS

PGG reduced activation of NF-κB and MAPK signalling pathways by directly interacting with the MyD88 adaptor protein. PGG may ameliorate inflammatory diseases such as colitis.

Abbreviations

GRC, *Rhus chinensis* MILL; IBD, inflammatory bowel disease; IKK, IκB kinase; iNOS, inducible NOS; IRAK, IL-1 receptor-associated kinase; MPO, myeloperoxidase; MyD88, myeloid differentiation primary response gene; PGG, penta-O-galloyl-β-D-glucose; TAK1, transforming growth factor-β-activated kinase-1; TLR, Toll-like receptor; TNBS, 2,3,4-trinitrobenzene sulphonic acid

Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, consists of chronically relapsing disorders of the intestine (Shanahan, 2002; Binder, 2004). The pathogenic mechanism was proposed to be dys-regulation of the intestinal immune response to environmental antigens in the gastrointestinal tract, such as intestinal microbiota that mainly reside in the ileum, caecum and colon (Rafii *et al.*, 1999; Atreya *et al.*, 2000; Joh *et al.*, 2011). Normal intestinal microbiota consist of more than 1000 bacterial species, which are directly influenced by endogenous and exogenous factors such as diet, drugs, stress and hormones (Peris-Bondia *et al.*, 2011; Kim *et al.*, 2012; Pendyala *et al.*, 2012). For example, a high-fat diet induces LPS, a known inflammatory mediator, in the intestinal contents of humans and animals, supporting that intestinal microbiota initiate and perpetuate colonic inflammation. More specifically, intestinal bacterial endotoxins penetrate the epithelial barrier and stimulate immune reactions in the mucosa (Radema *et al.*, 1991; Rafii *et al.*, 1999). Subsequently, these toxins induce production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and other inflammatory mediators, leading to inflammatory activation via distinct signalling pathways through Toll-like receptors (TLRs) and/or cytokine receptors (Jung *et al.*, 1995; Chow *et al.*, 1999; Cario and Podolsky, 2000; Joh and Kim, 2011; receptor nomenclature follows Alexander *et al.*, 2011). Suppressing the expression of these inflammatory mediators therefore decreases the incidence of inflammatory diseases. The assessment of natural products that may lessen inflammatory diseases has recently become a topic of much interest (Paradkar *et al.*, 2004; Joh and Kim, 2011).

The gallnut of *Rhus chinensis* MILL (GRC; family *Anacardiaceae*), which contains 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG) as a major constituent (Park *et al.*, 2008), is used for treating diarrhoea, excessive sweating, bleeding, and chronic coughs as a traditional Chinese medicine in China, Japan and Korea (Djakpo and Yao, 2010; Shim *et al.*, 2003; Kim *et al.*, 2005; Ren *et al.*, 2008). It is reported to inhibit α-glucosidase (Shim *et al.*, 2003) and to display anti-anaphylactic (Kim *et al.*, 2005), anti-viral (Nakano *et al.*, 1998) and anti-ischaemic activities (Lee *et al.*, 2012). PGG inhibits TNF-α and IL-6 secretion in LPS-stimulated peripheral blood mononuclear cells (Genfa *et al.*, 2005), and also reduces the TNF-α level in rats after systemic administration of LPS. Oh *et al.* (2004) reported that PGG blocks IL-8 mRNA expression and secretion in human monocytic U937 cells stimulated with phorbol 12-myristate 13-acetate (PMA) or TNF-α. Finally, PGG significantly inhibits COX-2 and inducible NOS (iNOS) expression, as well as NF-κB activation via inhibition of IκB kinase (IKK) activity in LPS-activated RAW264.7 cells (Pan *et al.*, 2000; Lee *et al.*, 2003).

Our long-standing search for anti-inflammatory agents from natural products showed that GRC potently inhibited the phosphorylation of IL-1 receptor-associated kinase 1 (IRAK1) phosphorylation and NF-κB activation in murine primary peritoneal macrophages, stimulated by LPS- or peptidoglycan (PGN). Recently, we additionally found that PGG, a major constituent of GRC, inhibited the activation of PI3K and MAPKs in LPS-stimulated peritoneal macrophages, which was not investigated in a previous similar report (Pan *et al.*, 2000). Therefore, in this study we attempted to clarify the anti-inflammatory mechanism of PGG by thoroughly investigating the molecular basis of its anti-inflammatory effect, using LPS-induced peritoneal and colonic macrophages, in a model of systemic inflammation induced by LPS and a model of colitis induced by 2,3,4-trinitrobenzene sulphonic acid (TNBS) in mice.

Methods

Animals

All animal care and experimental procedures were in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use, and were approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University (KHP-2012-11-01-R1). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 95 animals were used in the experiments described here. Male C57BL/6 mice (18–22 g, 6 weeks) were supplied from the Orient Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea) and allowed water *ad libitum*.

Isolation and culture of peritoneal and colonic macrophages

To isolate peritoneal macrophages, mice were intraperitoneally injected with 2 mL of 4% sodium thioglycolate. Mice were killed 4 days after injection, and the peritoneal cavities were flushed with 10 mL RPMI 1640. The peritoneal lavage fluids were centrifuged at 200× *g* for 10 min, and then the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37°C, the cells were washed three times and non-adherent cells were removed by aspiration. Cells were cultured in 24-well plates (5×10^5 cells per well) at 37°C in RPMI 1640 with 10% fetal bovine serum (FBS). Peritoneal macrophages were separated from colonic cells using a biotin-labeled anti-mouse F4/80 antibody (Invitrogen, Carlsbad, CA, USA) and streptavidin magnetic beads (Invitrogen).

To isolate macrophages from the intestine, Peyer's patches were isolated from mouse intestines and digested in DMEM containing 1 mg·mL^{-1} dispase, 0.25 mg·mL^{-1} collagenase A and 25 U·mL^{-1} DNAase (Roche Diagnostic, Indianapolis, IN, USA) at 37°C for 20 min with shaking, and then passed through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA). The cells were collected by centrifugation and then washed with PBS containing 4% FBS. The cells were resuspended in cold IMag buffer (0.05% BSA and 2 mM EDTA in PBS), and viable cells were counted by Trypan blue exclusion. Colonic macrophages were separated from colonic cells using a biotin-labelled anti-mouse F4/80 antibody and streptavidin magnetic beads (Invitrogen). To examine the anti-inflammatory effect of PGG, peritoneal or colonic macrophages were incubated in the absence or presence of PGG with 50 ng·mL^{-1} LPS or PGN.

Confocal and fluorescent microscopy

For the p65 assay, peritoneal macrophages were stimulated with LPS (100 ng·mL^{-1}) in the presence or absence of PGG for

60 min. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Then, the cells were stained with a goat anti-p65 polyclonal antibody for 2 h at 4°C, followed by incubation with Alexa 488-conjugated secondary antibody propidium iodide (10 μg·mL^{-1}; Calbiochem Co., San Diego, CA, USA) for 1 h. Images were obtained using confocal microscopy.

For the LPS-TLR4 complex assay, peritoneal macrophages plated on glass slides were incubated at 37°C overnight. Macrophages were stimulated with Alexa Fluor 594-conjugated LPS (10 μg·mL^{-1}; Invitrogen) for 20 min in the presence or absence of PGG. The cells were then fixed with 4% formaldehyde and 3% sucrose for 20 min (Baldwin, 1996; Joh *et al.*, 2012). Then, the cells were stained with a rabbit anti-TLR4 polyclonal antibody for 2 h at 4°C, followed by Alexa Fluor 488-conjugated secondary antibodies for 1 h (Park *et al.*, 2008). The stained cells were then observed by confocal microscopy.

For MyD88 and IRAK assays, peritoneal macrophages were stimulated with LPS (100 ng·mL^{-1}) in the presence or absence of PGG for 30 min. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Then, the cells were stained with goat anti-MyD88, anti-IRAK1, or anti-p-IRAK1 polyclonal antibody for 2 h at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody for 1 h (Park *et al.*, 2008). The stained cells were observed by confocal microscopy.

Flow cytometry

Mouse peritoneal macrophages were incubated with Alexa Fluor 488-conjugated LPS (10 μg·mL^{-1}) for 10 min at room temperature. The cells were then fixed in PBS containing 4% paraformaldehyde and 3% sucrose for 20 min. After washing with PBS, the macrophages were incubated with propidium iodide (10 μg·mL^{-1}) for 10 min and then analysed by flow cytometry.

Transient transfection with siRNA

Cells (3×10^5 cells per well) were seeded in 24-well plates and allowed to rest for 24 h. Then the cells were transfected with 100 nM siRNA for TLR4 or MyD88 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction. At 48 h after transfections, cells were treated with or without PGG (5 μM) and/or LPS (50 ng·mL^{-1}).

Model of colitis in mice

Mice were randomly divided into five groups: normal (control) and TNBS-induced colitis groups treated with or without PGG or mesalazine. Each group consisted of seven mice. Colitis was induced by slow intrarectal administration of a 2.5% (w/v) TNBS solution (100 μL) in 50% ethanol into the colon of anaesthetized mice via a thin round-tip needle attached to a 1 mL syringe (Joh *et al.*, 2011). The normal (control) group was treated with the vehicle alone. The needle was inserted so that the tip was 3.5–4.0 cm proximal to the anal verge. To distribute the agents within the entire colon and caecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS-ethanol solution, it was excluded from the remainder of the study. PGG (5 or 10 mg·kg^{-1}) [based on the ethnopharmacological dose (2–10 g GRC/day/person) and the preliminary study] or mesalazine (10 mg·kg^{-1}) dissolved in 2% Tween 80 was orally administered once a day for 3 days after TNBS treatment. The mice were killed the day after the final administration of PGG or mesalazine. The colon was quickly removed, opened longitudinally and gently cleared of stool by washing with PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulceration and no inflammation; 1, no ulceration and local hyperaemia; 2, ulceration with hyperaemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm), and the colon tissue was divided longitudinally for immunoblotting, ELISA, myeloperoxidase (MPO) and histological examination.

For the assays of immunoblotting, ELISA, and MPO activity, the colon was homogenized in 1 mL ice-cold RIPA containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The homogenates were centrifuged (15 000× *g*, 4°C) for 15 min, and then the supernatants were collected and used.

For the histological studies, one longitudinal part of each colon was fixed overnight in 50 mM phosphate buffer (pH 7.4) containing 4% paraformaldehyde, and then immersed in a 30% sucrose solution (in 50 mM phosphate buffered saline) and stored at 4°C until sectioning. Frozen colons were sectioned along the coronal plane (10 μm) using a cryostat (Leica Microsystems, AG, Germany), and stained with haematoxylin–eosin.

MPO activity assay

An aliquot (50 μL) of the colon supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2, and then incubated at 37°C. The absorbance was obtained at 650 nm over time. MPO activity was defined as the quantity of enzyme degrading 1 μmol·mL^{-1} of peroxide at 37°C, and expressed in unit·(mg protein)$^{-1}$ (Joh *et al.*, 2011).

ELISA and immunoblotting

For the ELISAs of IL-1β, IL-6, IL-10 and TNF-α, colons or cell culture supernatants were transferred to 96-well ELISA plates. IL-1β, IL-6, IL-10 and TNF-α concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, USA).

For immunoblot analyses of p-IRAK1, p-IKKβ, p-p65 and β-actin, colon tissue homogenates or collected cells were resuspended in 1 mL RIPA containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was used for the immunoblot assay. The proteins from collected cells were separated by electrophoresis on an 8–10% SDS–polyacrylamide gel, and then transferred to a nitrocellulose membrane and immunoblotted using standard procedures (Joh *et al.*, 2011).

Data analysis

All data are expressed as the mean ± SD. Statistical significance was assessed using one-way ANOVA followed by the Student's *t* Newman–Keuls test.

Materials

LPS was purified from *Escherichia coli* O111:B4, PGN was purified from *Staphylococcus aureus* cell wall component. TNBS, DMEM, RPMI 1640 and mesalazine were purchased from Sigma Co. (St. Louis, MO, USA). Antibodies for MyD88 (sc-8197), IRAK1 (sc-7883), p-IRAK1 (sc-130197), IRAK2 (sc-23652), IRAK4 (sc-34470), IκBα (sc-371), p65 (sc-372), COX-2 (sc-1747-R), iNOS (sc-650), β-actin (sc-47778) and small–interfering RNA (siRNA) for MyD88 and for TLR4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for TLR4 (2219S), p-p65 (3033 L), p-IκBα (2859S), p-IKKβ (2078S), transforming growth factor-β-activated kinase-1 (TAK1; 4505S), p-TAK1 (9339S), ERK (4695S), p-ERK (9101 L), JNK (9252S), p-JNK (9251S), p38 (9211 L), p38 (9212) were purchased from Cell Signaling Technology (Beverly, MA, USA). All antibodies were used at a dilution of 1:1000. ELISA kits for cytokines and PGE_2 were purchased from R&D Systems (Minneapolis, MN, USA). PGG (purity, >95%) was isolated from 80% EtOH extract of GRC as previously reported (Park *et al.*, 2008).

Results

PGG reduces the phosphorylation of IRAK1 and p65 under LPS- and PGN-stimulated conditions in macrophages

As PGG is known to be a major active constituent of GRC, we purified PGG and tested it in LPS- or PGN-stimulated peritoneal macrophages (Figure 1A) and colonic macrophages (Figure 1B). In both LPS- or PGN-stimulated macrophage populations, PGG (5 μM) strongly decreased the phosphorylation of IRAK1 and p65, an indicator of NF-κB activation. These results are consistent with our preliminary data using ethanol extracts of GRC (Supporting Information Figure S1). We then assessed cytotoxicity of PGG and found that exposure to 20 μM of PGG for 48 h did not affect the viability of macrophages (Figure 1C).

PGG treatment reduces the phosphorylation of several signalling molecules

Using LPS-stimulated peritoneal macrophages, we investigated the effects of PGG on several intracellular signalling proteins. As shown in Figure 2A, LPS treatment reduced the expression of IRAK2 and IRAK4 expression in peritoneal macrophages and this was prevented by co-treatment with PGG. LPS treatment also increased p-TAK1, p-IKKβ and p-IκBα and these changes were prevented by PGG (5 μM). LPS treatment increased levels of p-ERK, p-JNK and p-p38, components of the MAPK signalling pathway. We found that co-treatment with PGG concentration-dependently reduced p-ERK and p-p38 levels, while only 5 μM of PGG decreased p-JNK. It should be noted that even at the highest PGG concentration, the phosphorylation levels of all three components of the MAPK pathway were still above control levels, suggesting that PGG could not completely prevent this signalling pathway of macrophages from responding to LPS.

Because PGG inhibited the induction of p-p65, we examined the cellular localization of total p65 during different treatment conditions. Macrophages were left untreated or with LPS alone or with LPS and PGG. Next the cells were fixed and examined for the intracellular location of p65. As showing in Figure 2C, LPS caused p65 to move into the nucleus (DAPI stained). However, PGG decreased the p65 localization in the nucleus of LPS co-treated macrophages. Collectively, these data further support our immunoblot data showing that PGG inhibited activation of NF-κB in macrophages.

To further characterize the effect of PGG on LPS-stimulated macrophages, we assessed a range of cytokines. LPS induced production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Figure 3A–C). Co-treatment with PGG and LPS produced lower levels of these cytokines, compared with those after to LPS alone. Also, LPS treatment reduced production of IL-10, while PGG treatment restored the production of this cytokine (Figure 3D), as observed earlier for echinocystic acid (Joh *et al.*, 2011). We also measured PGE_2, another known mediator of inflammation. PGE_2 was significantly increased with LPS treatment and PGG co-treatment reduced this increase (Figure 3E). Production of NO (measured as nitrite) by macrophages was significantly increased by LPS and this production was inhibited by co-treatment with PGG (Figure 3F). Finally, immunoblot analysis showed that COX-2 and iNOS levels were induced by LPS treatment and PGG concentration-dependently reduced these levels. All these data indicate that PGG treatment reduced levels of several pro-inflammatory molecules.

Next, to clarify the mechanism of inhibition by PGG in LPS-stimulated peritoneal macrophages, we examined whether PGG could block the interaction between LPS and TLR4. As shown in Figure 4A, basal autofluorescence was determined in untreated peritoneal macrophages. Other samples were treated with Alexa Fluor 488-conjugated LPS alone or with different concentrations of PGG plus Alexa Fluor 488-conjugated LPS. PGG did not block LPS binding to the macrophage as indicated by a similar shift to the right in fluorescence (percentages show shift from baseline). We further confirmed our FACS assay-based observation by confocal microscopy (Figure 4B) to show that PGG did not block the binding of LPS to TLR4 on the cell surface. We also showed by immunoblot analysis that LPS treatment caused an increase in TLR4 expression and PGG treatment did not diminish this LPS-induced increase (Figure 4C).

We next examined some intracellular effectors that are associated with TLR4 and are upstream of NF-κB. Macrophages were treated with siRNA for MyD88, plus LPS and PGG. We then monitored the cellular localization of MyD88, IRAK4, p-IRAK1 and IRAK1 (Figure 5). Treatment with MyD88 siRNA reduced MyD88 levels in the cell (Figure 5A) and PGG plus LPS treatment also decreased MyD88 levels in cells without MyD88 siRNA, a result which may be due to PGG blocking the MyD88 antibody from binding or to an actual decrease in MyD88 expression. Expression of IRAK4 was increased by MyD88 siRNA treatment, regardless of LPS or PGG treatment, compared with controls (Figure 5B). Moreover, LPS treatment also decreased the levels of IRAK4, consistent with data in Figure 2A. The presence of p-IRAK1 was markedly increased in the nucleus of LPS-treated macrophages, an effect reduced by PGG. Cells treated with MyD88 siRNA did not show similar accumulation of p-IRAK1 (Figure 5C). When total IRAK1 was examined (Figure 5D), no significant change in intensity within the cells was observed

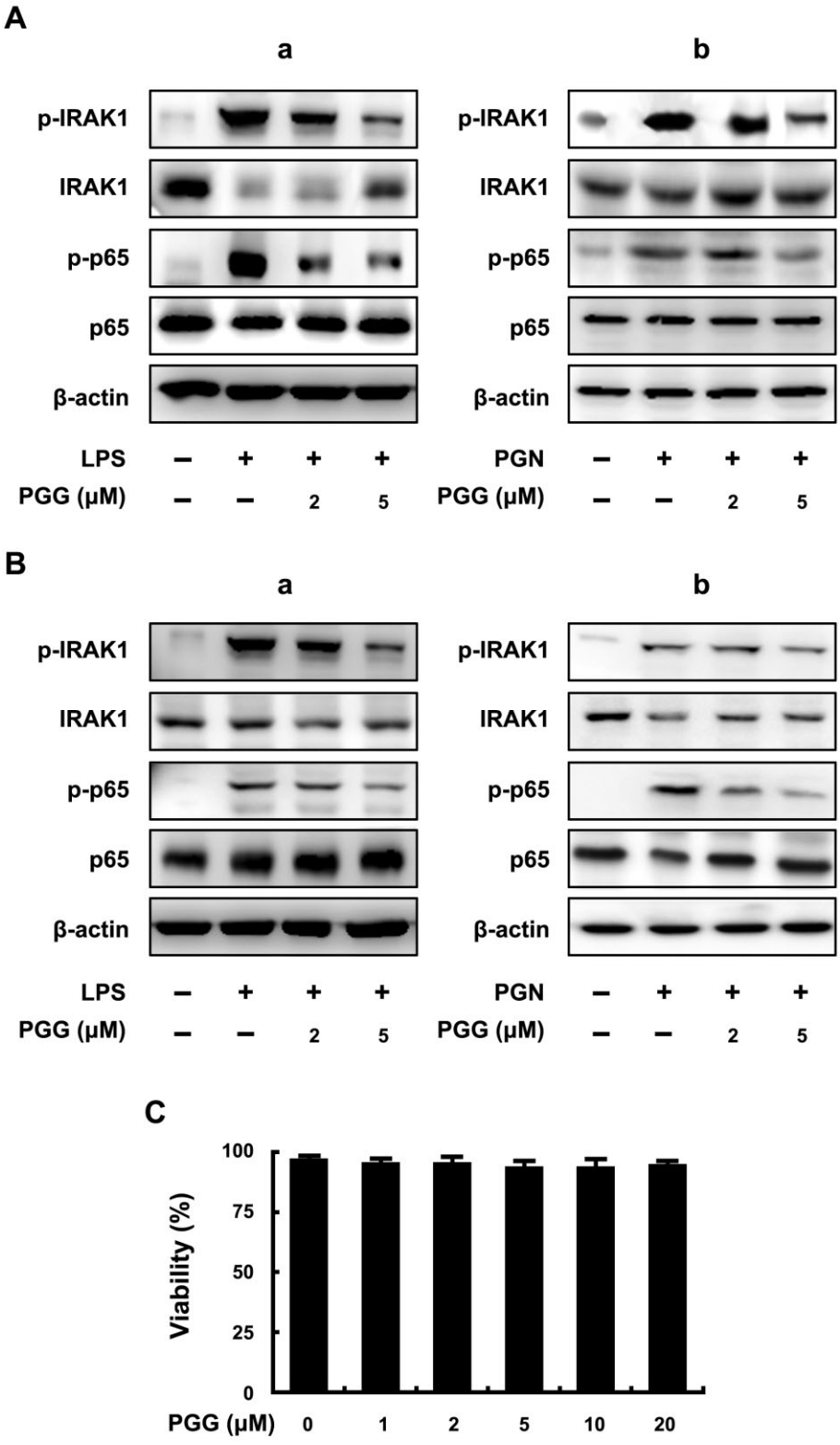

Figure 1

Effect of PGG on IRAK1 phosphorylation and NF-κB activation in LPS- or PGN-stimulated peritoneal and colonic macrophages. (A) Effect in peritoneal macrophages stimulated with LPS (a) or PGN (b). (B) Effect in colonic macrophages stimulated with LPS (a) or PGN (b). Peritoneal macrophages (5×10^5 cells) were treated with 50 ng·mL^{-1} LPS in the absence or presence of PGG (2 or 5 μM) for 20 h. IRAK1, p-IRAK1, p65, p-p65 and β-actin levels were measured by immunoblotting. (C) Cytotoxicity in peritoneal macrophages. Peritoneal macrophages (5×10^5 cells) were treated with PGG (2, 5 and 20 μM for 48 h.

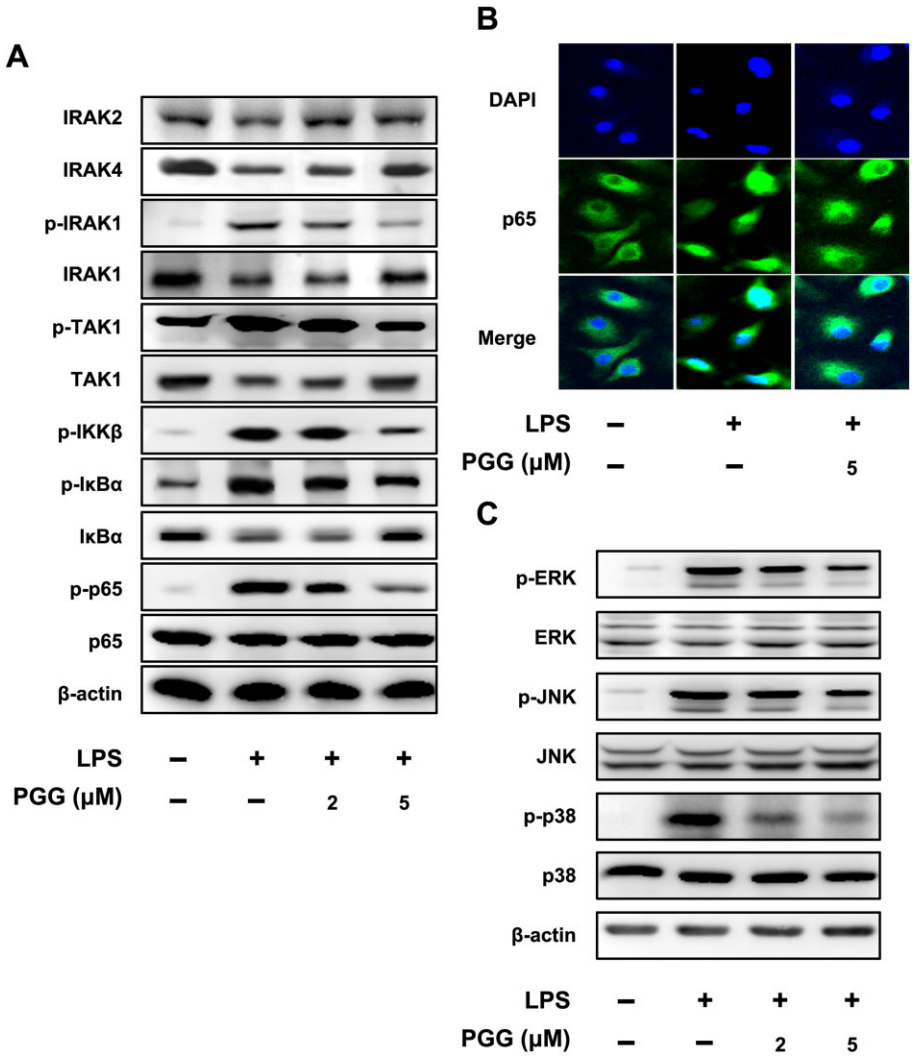

Figure 2

Inhibitory effect of PGG on NF-κB and MAPK signalling pathways in LPS-stimulated peritoneal macrophages. Peritoneal macrophages were treated with 50 ng·mL^{-1} LPS in the absence or presence of PGG (2 or 5 μM). (A) Effect on IRAK1/NF-κB pathway. (B) Effect on NF-κB nuclear translocation, as detected by confocal microscopy using an antibody against p65 subunit. (C) Effect on MAPK pathway. NF-κB and MAPK phosphorylation as well as nuclear translocation of NF-κB in LPS-stimulated peritoneal macrophages were determined at 90 min after treatment with LPS by immunoblotting and confocal microscopy respectively.

for the different treatment groups. PGG (5 and 10 μM) did not inhibit IRAK-1 kinase activity *in vitro*, assayed according to our already described method (Joh *et al.*, 2011; data not shown).

To further confirm the results from confocal microscopy, we performed immunoblot analysis for MyD88, IRAK4, p-IRAK1 and IRAK. LPS treatment alone did not influence MyD88 expression. However, IRAK4 expression was decreased and p-IRAK1 expression was increased. PGG plus LPS co-treated macrophages did not significantly influence MyD88 expression, but showed more normalization of IRAK4, p-IRAK1 and IRAK1 levels. MyD88 siRNA macrophages showed no significant change in MyD88 levels for all the groups. Further, we observed that LPS alone or LPS plus PGG treatments did not significantly change the levels of IRAK4, p-IRAK1 and IRAK1. Collectively, these data demonstrate the treatment with PGG may directly influence MyD88 levels in activated macrophages. Alternatively, PGG may bind to the MyD88 adaptor protein and block its interactions with other signalling molecules.

Inhibitory effect of PGG on TNBS-induced colitis in mice

We then investigated the effects of PGG on another *in vivo* model of inflammation, TNBS-induced colitis in mice. TNBS caused severe colonic inflammation, assayed as colon shortening, increased MPO activity, NF-κB activation and IRAK1 phosphorylation. Treatment with PGG or mesalazine significantly reduced TNBS-induced weight loss (Figure 6A), macroscopic disease (Figure 6A), and shortening of the colon (Figure 6C), and MPO activity (Figure 6D). Macroscopic images of the colons are shown in Figure 6E and microscopic examinations of sections stained with haematoxylin–eosin

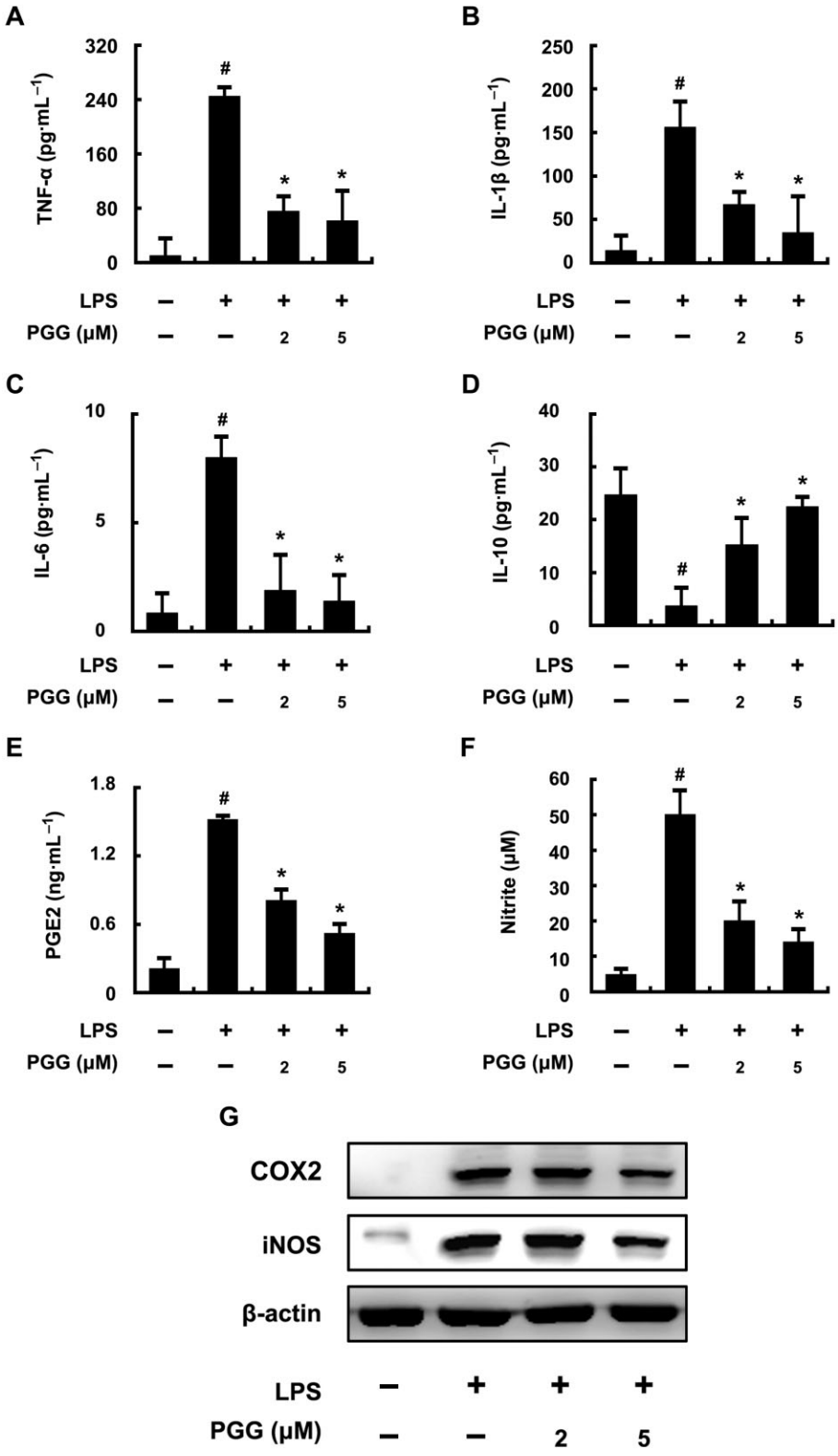

Figure 3

Inhibitory effect of PGG on inflammatory markers in LPS-stimulated peritoneal macrophages. Peritoneal macrophages (5×10^5 cells) were treated with 50 ng·mL^{-1} LPS in the absence or presence of PGG (2 or 5 μM) for 20 h. The levels of TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D), PGE_2 (E) and NO (F) in culture supernatants were measured by ELISA. COX-2 and iNOS levels (G) in the supernatants of lysed cells were measured by immunoblotting. All data are expressed as the mean ± SD ($n = 4$ in a single experiment). $^{\#}P < 0.05$ significantly different from control group; $^{*}P < 0.05$, significantly different from LPS alone.

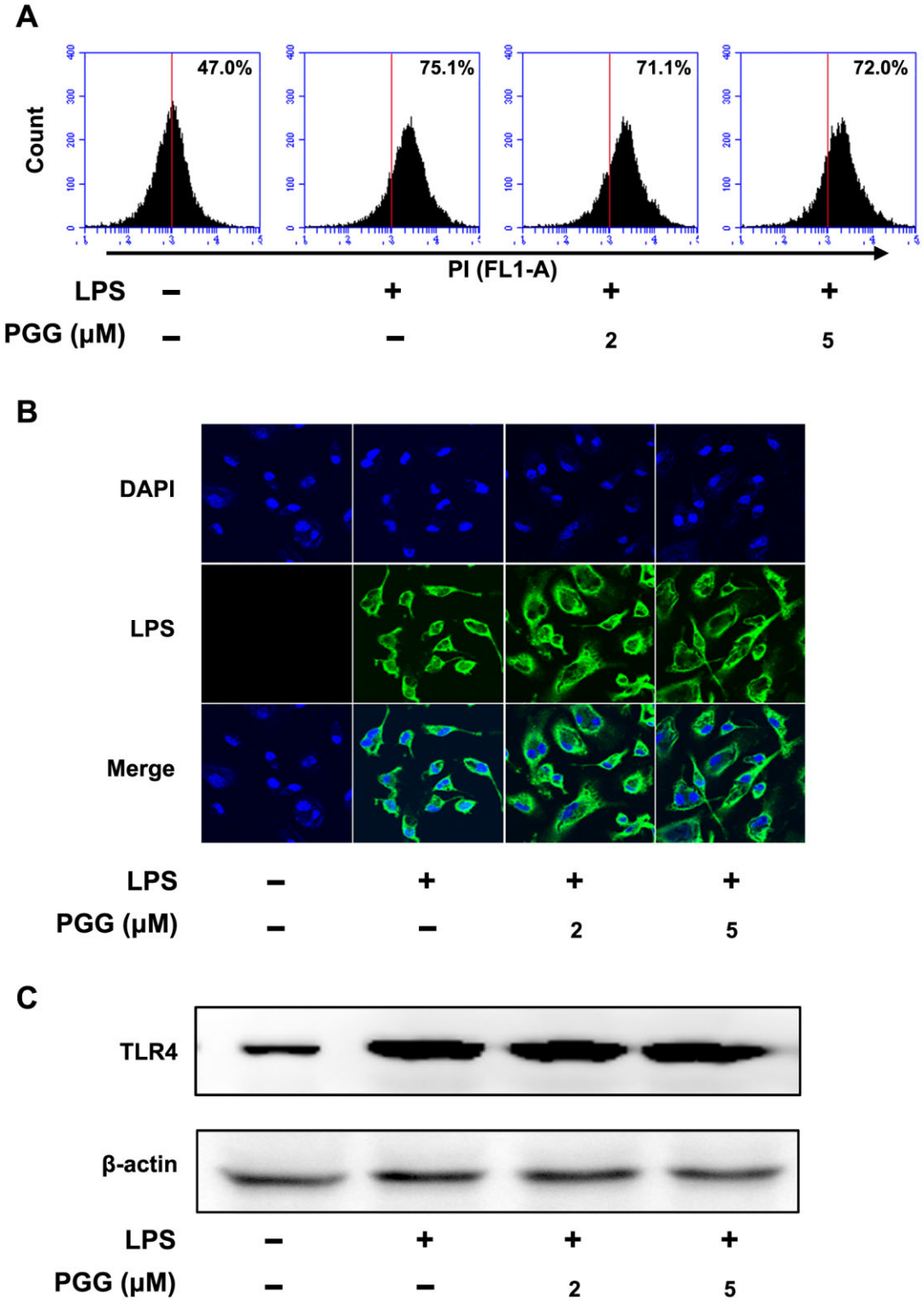

Figure 4

Effect of PGG on the binding of LPS to TLR-4 on peritoneal macrophages. Peritoneal macrophages (5×10^5 cells) isolated from mice were incubated with Alexa Fluor 488-conjugated LPS for 20 min in the absence or presence of PGG (2 or 5 μM), and then analysed by flow cytometry (A), confocal microscopy (B), and immunoblotting (C). TLR4 and β-actin were analysed by immunoblotting.

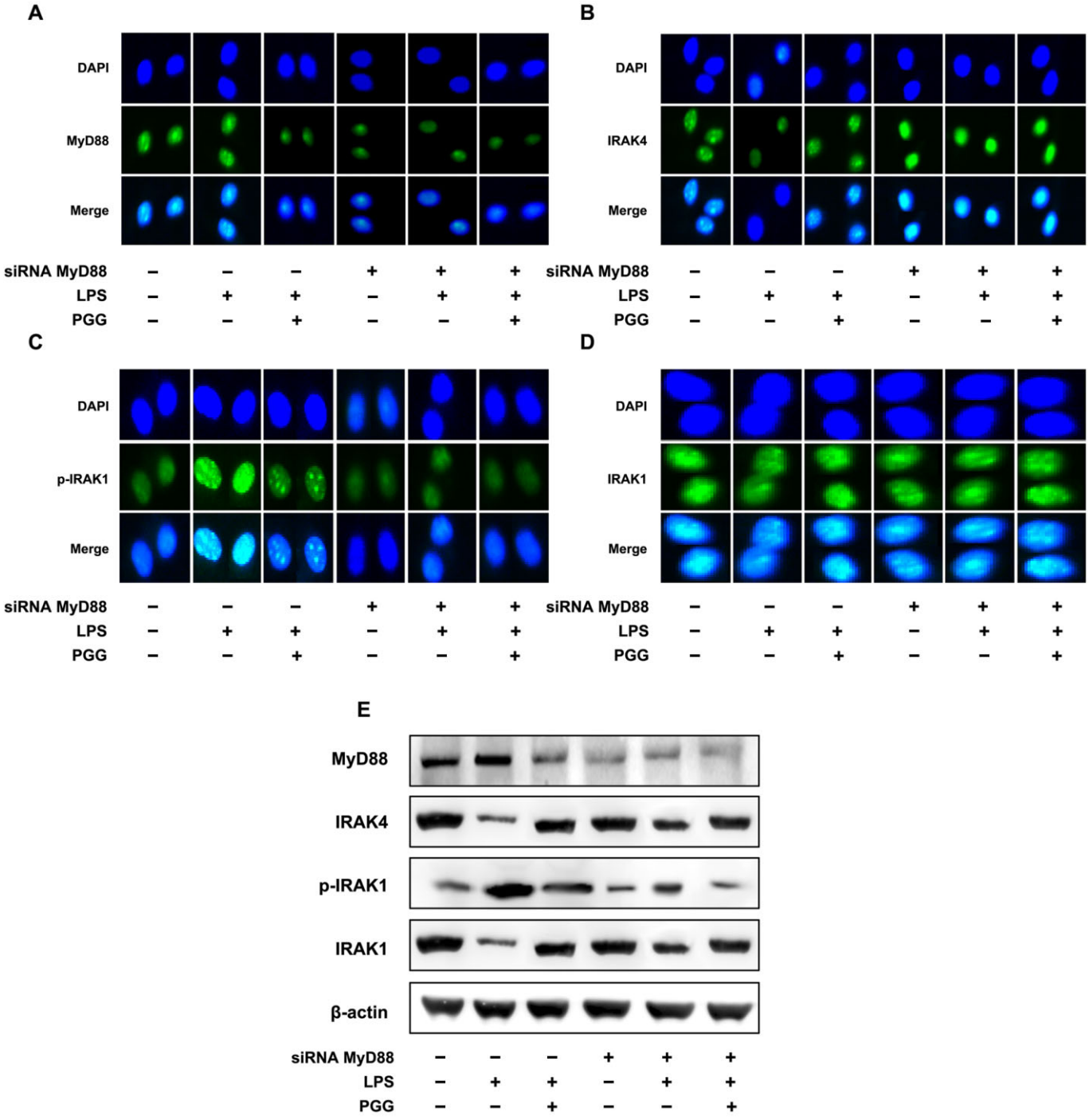

Figure 5

Inhibitory effect of PGG on IRAK1 and NF-κB activation in LPS-stimulated peritoneal macrophages transfected with MyD88 siRNA. (A) Effect on MyD88 expression. (B) Effect on IRAK4 expression. (C) Effect on p-IRAK1 expression. (D) Effect on IRAK1 expression. Peritoneal macrophages isolated from mice were treated with 50 ng·mL^{-1} LPS in the absence or presence of PGG (5 μM) for 90 min. Alexa Fluor 488-conjugated IRAK1 and p-IRAK1 were detected in siRNA-transfected peritoneal macrophages. (E) Effect on IRAK1 and IRAK4 degradation and IRAK1 phosphorylation. MyD88, IRAK1, p-IRAK1, IRAK4 and β-actin were analysed by immunoblotting. Peritoneal macrophages were treated with 50 ng·mL^{-1} LPS in the absence or presence of PGG (5 μM) for 90 min.

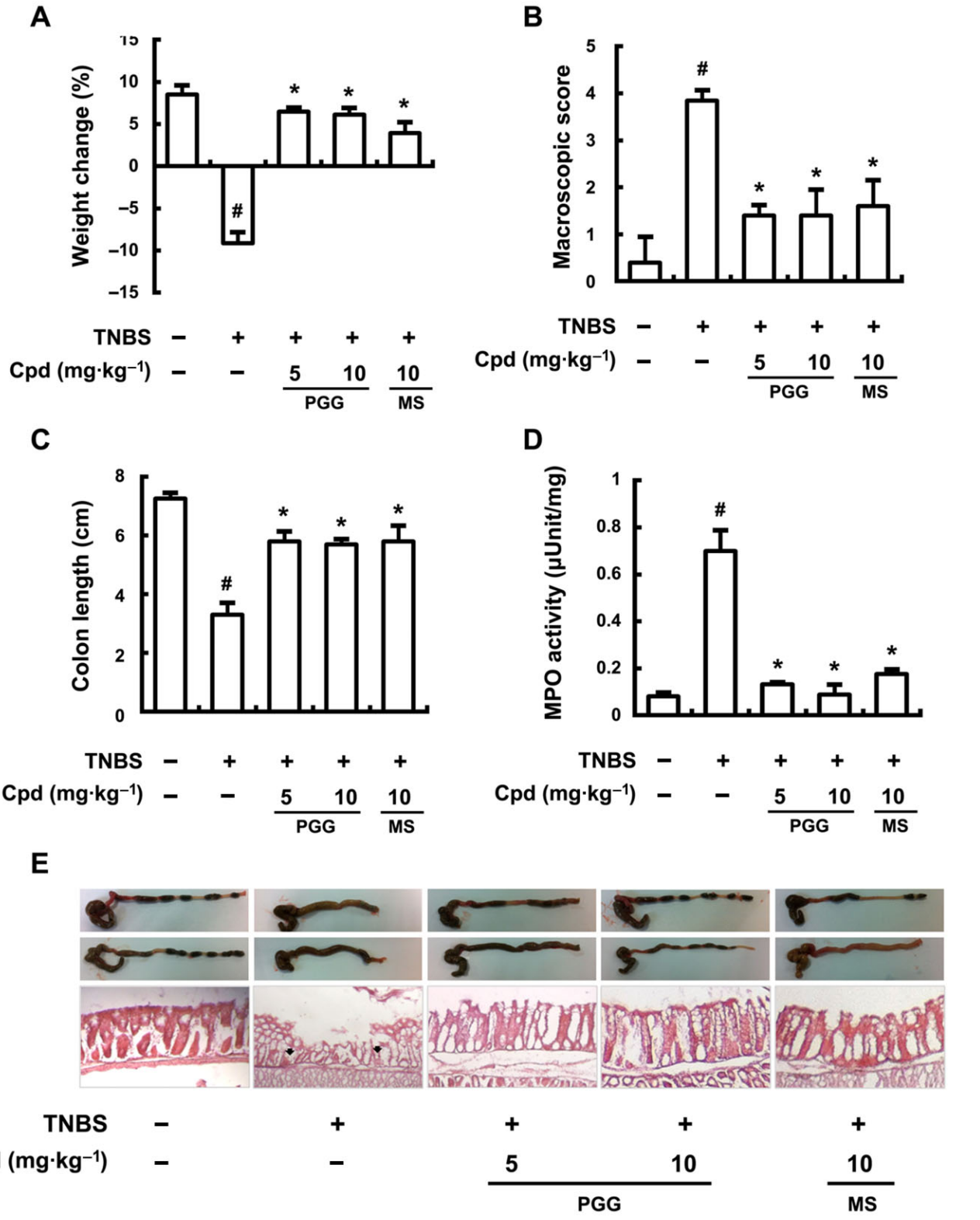

Figure 6

Effect of PGG on body weight (A), macroscopic disease (B), colon length (C), colonic MPO activity (D), and histology (haematoxylin–eosin staining) (E) in mice with TNBS-induced colitis. TNBS, except in the control group, was intrarectally administered to mice treated with saline, PGG or mesalazine. Test compounds [Cpd; PGG (5 or 10 mg·kg^{-1}), mesalazine (MS; 10 mg·kg^{-1}), or saline] were orally administered for 3 days after TNBS treatment. The mice were killed at 20 h after the final administration of PGG or mesalazine. All values are the mean ± SD ($n = 7$). $^{\#}P < 0.05$, significantly different from control group; $^{*}P < 0.05$, significantly different from TNBS alone.

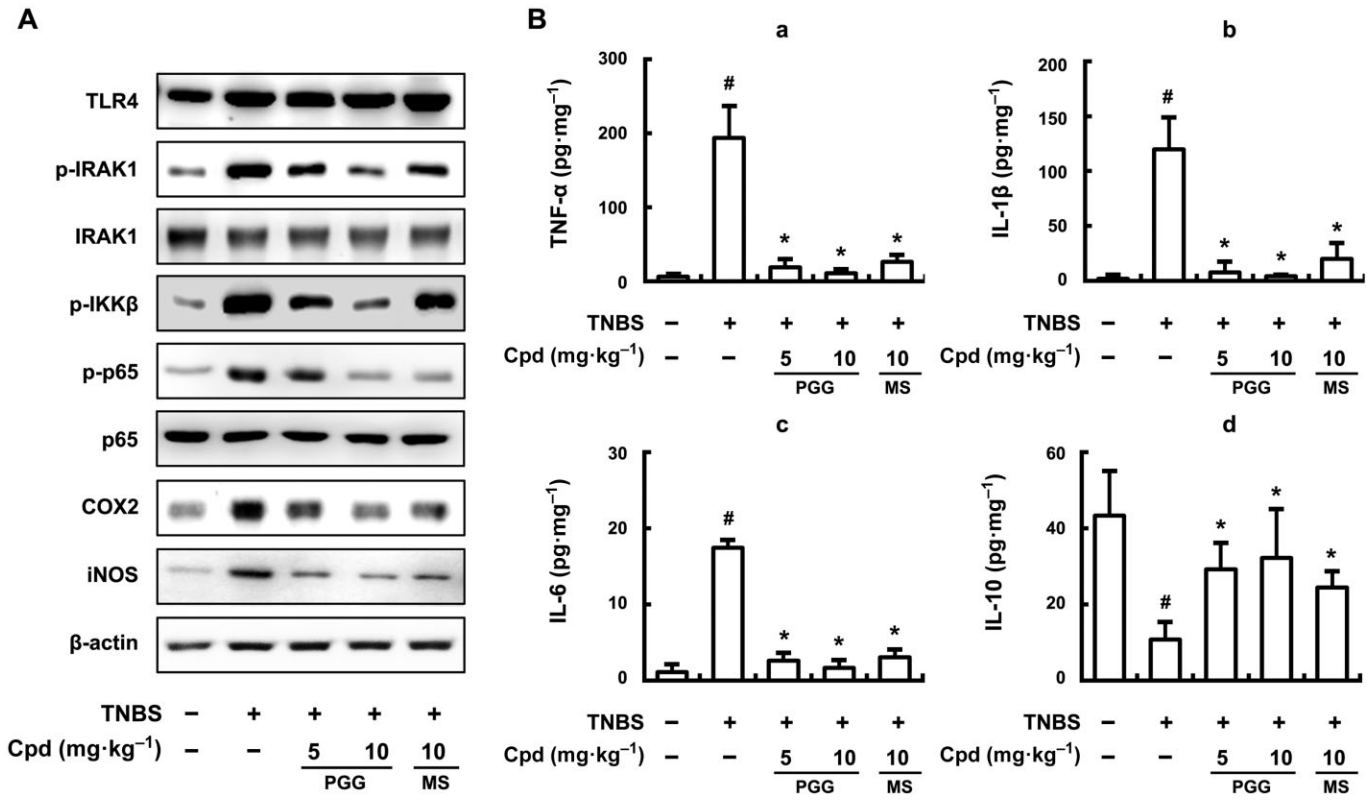

Figure 7

Effect of PGG on phosphorylation of IRAK1 and p65 and expression of iNOS, COX-2 (A), and proinflammatory cytokines (B) in mice with TNBS-induced colitis. TNBS, except in the control group, was intrarectally administered to mice treated with saline, PGG or mesalazine. PGG (5 or 10 mg·kg^{-1}), mesalazine (MS, 10 mg·kg^{-1}), or saline was orally administered for 3 days after TNBS treatment. The mice were killed at 20 h after the final administration of test compound (Cpd; PGG or mesalazine). (A) TLR4, IRAK1, p-IRAK1, IKKβ, p-IKKβ, COX-2, iNOS and β-actin were analysed by immunoblotting. (B) TNF-α (a), IL-1β (b), IL-6 (c), IL-10 (d) were analysed by ELISA kits. All values are the mean ± SD ($n = 7$). $^{\#}P < 0.05$, significantly different from control group; $^{*}P < 0.05$, significantly different from TNBS alone.

are displayed below for the different treatment groups. The anti-colitis effects of PGG were comparable to those of that of mesalazine (10 mg·kg^{-1}).

Next we monitored protein expression levels after different treatments. Although TLR4 levels were not changed in the different treatment groups (Figure 7A), TNBS treatment did increase p-IRAK1, p-IKKβ, p-p65, COX-2 and iNOS levels. Both PGG and mesalzine reduced the levels of p-IRAK1, p-IKKβ and p-p65 proteins, and also inhibited the expression of COX-2 and iNOS. Furthermore, PGG (10 mg·kg^{-1}) almost totally blocked the increase in TNF-α, IL-1β and IL-6 expression in mice with TNBS-induced colitis (Figure 7B). However, PGG restored the levels of IL-10, which had been depressed by TNBS, to nearly normal values. Nevertheless, PGG, at a much higher dose (25 mg·kg^{-1}), did not show acute toxicity (body weight, abnormal behaviour and death) when observed for 7 days under the conditions used in the experiment (data not shown).

Discussion

A number of *in vitro* and *in vivo* studies have shown that PGG exhibits a wide range of biological activities in the therapy and prevention of several major human diseases including cancer, diabetes and inflammation (Zhang *et al.*, 2009). PGG inhibits the expression of TNF-α in LPS-stimulated human peripheral blood mononuclear cells (Feldman *et al.*, 2001) and the secretion of TNF-α and IL-6 induced by IL-1β in rat synoviocytes (Wu and Gu, 2009). PGG also inhibits IL-8 mRNA expression and secretion in human monocytic U937 cells stimulated with PMA or TNF-α (Lee *et al.*, 2007). PGG strongly suppresses PMA and calcium ionophore A23187 induced expression of TNF-α, IL-1β and IL-6 in human mast cells (Lee *et al.*, 2007). In addition, PGG significantly inhibits COX-2 and iNOS activity, as well as NO production in LPS-stimulated RAW264.7 cells (Lee *et al.*, 2003). PGG inhibits iNOS and NADPH-diaphorase activities and NO release in LPS-induced peritoneal macrophages (Yokozawa *et al.*, 2000), and significantly reduces serum TNF-α level in LPS-stimulated endotoxaemic mice (Genfa *et al.*, 2005). The anti-inflammatory effect of PGG may be due to inhibition of the phosphorylation of IκB in the cytosol, which inhibits NF-κB activation in LPS-activated RAW264.7 cells (Pan *et al.*, 2000).

In the present study, we observed that PGG exhibited an anti-inflammatory effect in LPS- or PGN-induced peritoneal macrophages and in LPS-induced colonic macrophages. PGG also potently inhibited the expression levels of COX-2 and

iNOS as well as their products, PGE_2 and NO, in LPS-treated peritoneal macrophages, mechanistically explaining the previously observed inhibitory effect of PGG against COX-2 and iNOS activity (Lee *et al.*, 2003). Furthermore, LPS induced expression of pro-inflammatory cytokines as previously reported (Brightbill *et al.*, 2000; Cao *et al.*, 2005; Joh and Kim, 2011). However, in our experiments, LPS reduced IL-10 expression, in contrast to earlier reports showing that LPS increased IL-10 expression in macrophages (Brightbill *et al.*, 2000; Cao *et al.*, 2005; Soudi *et al.*, 2013). This discrepancy should be resolved by further study. PGG inhibited the LPS-induced expression of pro-inflammatory cytokines TNF-α IL-1β and IL-6, but increased the LPS-reduced IL-10 expression, results similar to those of other anti-inflammatory agents (Dutra *et al.*, 2011; Clemente *et al.*, 2012).

In terms of intracellular signalling, PGG inhibited LPS-induced IRAK1 degradation and phosphorylation, IKKβ phosphorylation, IκBα degradation and translocation of the p65 subunit of NF-κB into the nucleus. Furthermore, PGG inhibited the phosphorylation of MAPKs, JNK, ERK and p38, even though PGG did not inhibit the interaction between LPS and its receptor, TLR4, on the cell membrane of peritoneal macrophages. Interestingly, PGG inhibited binding of a fluorescent MyD88 antibody to peritoneal macrophages and the fluorescent anti-MyD88 antibody was also not bound to peritoneal macrophages treated with MyD88 siRNA. Finally, this study also revealed that PGG did not inhibit the binding of fluorescent anti-IRAK1 and IRAK4 antibodies to peritoneal macrophages, irrespective of transfection with MyD88 siRNA. Therefore, these results suggest that PGG, by binding directly to MyD88, may inhibit the signalling from TLRs activated by LPS or PGN, normally transferred by MyD88 on to IRAK1.

In humans, IBD is a severe form of intestinal inflammation, the pathogenesis of which remains to be clearly understood. It is thought that the disease might be attributed to complex mucosal immune responses to resident enteric bacteria (Duchmann *et al.*, 1995; Jung *et al.*, 1995). The innate immune system recognizes the presence of specific bacterial antigens through pattern recognition receptors (Ingalls *et al.*, 1999). TLRs, which serve as a major link between the innate and adaptive mucosal immune responses, act as transmembrane co-receptors in the cellular response to pathogen antigens. Of the TLRs, TLR2 and TLR4 are pattern recognition receptors that respond to PGN (isolated from Gram-positive bacteria) and LPS (isolated from Gram-negative bacteria) respectively (Chow *et al.*, 1999; Ingalls *et al.*, 1999; Maldonado-Bernal *et al.*, 2005). These TLRs activate the secretion of pro-inflammatory mediators from monocytes, macrophages and dendritic cells, thereby triggering the activation of the acquired immune response. Particularly, TLR4 is potently expressed in intestinal epithelial cells from the colons of patients with IBD (Cario and Podolsky, 2000) and significantly up-regulated in mice with experimental colitis (Pasternak *et al.*, 2010). LPS may potentially activate the TLR4-NF-κB signalling pathway in IBD (Chow *et al.*, 1999; Pasternak *et al.*, 2010). The activation of NF-κB in mucosal macrophages is accompanied by an increased capacity of these cells to produce and secrete IL-1, IL-6 and TNF-α, which activates NF-κB. The adapter protein, MyD88, is used by all TLRs (except TLR3) as a link to the pathway of NF-κB activation (Kawai and Akira, 2007; Cario and Podolsky, 2000). TIRAP (Toll-IL 1 receptor domain-containing adapter protein) is necessary to recruit MyD88 to TLR2 and TLR4, and then MyD88 signals through IRAKs. Thus, binding of LPS or PGN to TLRs induces phosphorylation of IRAK1, PI3K and/or MAPKs, which are involved in host defence mechanisms, either by identification of pathogens or as receptors for pro-inflammatory cytokines.

Various inflammatory diseases, including colitis and systemic inflammation, involve the over-expression of pro-inflammatory cytokines such as TNF-α and IL-1β, and inflammatory mediators, such as NO and PGE_2 via NF-κB and MAPK pathways in macrophages (Tak and Firestein, 2001; Moynagh, 2005). Similar to the effects of kalopanaxsaponin A (Joh and Kim, 2011), compound K (Joh *et al.*, 2011) and echinocystic acid, PGG inhibited MAPK, IRAK/ NF-κB and PI3K-AKT pathways. However, the anti-inflammatory mechanism of PGG was different to those of kalopanaxsaponin A, compound K and echinocystic acid. Indeed, PGG inhibited activation of PI3K/AKT, NF-κB and MAPKs in macrophages, by preventing the signals from LPS- or PGN-bound TLRs to be transferred to IRAKs via MyD88.

In the model of colitis induced by TNBS in mice, PGG suppressed the macroscopic features of the colitis such as weight loss and colon shortening, and intracellular signalling events such as the activation of NF-κB and phosphorylation of IKKβ, IκBα and IRAK1. Furthermore, PGG also inhibited the expression of pro-inflammatory cytokines. In our model we used a lower dose of TNBS (2.5 mg per mouse), compared with earlier models (3–10 mg per mouse; Ukil *et al.*, 2006; Okamoto *et al.*, 2013) and found that the anti-colitis effect of PGG (10 $mg \cdot kg^{-1}$) was comparable to that of mesalazine (10 $mg \cdot kg^{-1}$). Mesalazine is used clinically at oral doses of 10–70 $mg \cdot kg^{-1}$ per day in patients with IBD (Das *et al.*, 1973; Sandborn and Hanauer, 2003). In many studies of experimental colitis in mice, mesalazine is given at oral doses of 50 or 100 $mg \cdot kg^{-1}$ (Malekinejad *et al.*, 2013; Sann *et al.*, 2013). However, in our preliminary studies, when we gave mesalazine at 10, 20 or 50 $mg \cdot kg^{-1}$ to our model of colitis in mice, we could not find any significant difference of anti-colitis effects such as colon shortening and macroscopic score between these doses (data not shown).

In conclusion, our results suggest, overall, that PGG may inhibit colitis by interfering with the TLR4-MyD88 signalling pathway. Our finding that PGG also reduced mortality, along with reduced expression of pro-inflammatory cytokines, in mice treated with LPS in a model of systemic inflammation (Supporting Information Figure S2), would provide additional support for this suggested mode of action of PGG. Based on these findings, we conclude that PGG may ameliorate inflammation by inhibiting MyD88 signalling pathways to NF-κB and/or MAPK.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effect of GRC on IKKβ phosphorylation, IκB-α degradation and NF-κB activation in LPS-stimulated peritoneal and colonic macrophages. (A) Effect in peritoneal macrophages stimulated with LPS. (B) Effect in colonic macrophages stimulated with LPS. Peritoneal macrophages (5×10^5 cells) were treated with 50 ng·mL^{-1} LPS in the absence or presence of GRC (5 or 10 μg·mL^{-1}) for 20 h. p-IKKβ, I, IκB-α, p-IκB-α, p65, p-p65 and β-actin levels were measured by immunoblotting.

Figure S2 Inhibitory effect of PGG on serum IL-1β and TNF-α levels and death of mice injected i.p. with LPS. (A) Effect on IL-1β (a) and TNF-α production (b). Mice were injected i.p. with or without PGG (5 or 10 mg·kg^{-1}) in the presence or absence of LPS (25 mg·kg^{-1}). The normal, control group was treated with vehicle alone instead of LPS and PGG. Mice were killed at 12 h after LPS injection and serum levels of IL-1β and TNF-α were measured using ELISA kits. (B) Effect on mortality. Septic shock was induced by injection of LPS (25 mg·kg^{-1}, i.p.) at 1 h after treatment with PGG (10 mg·kg^{-1}). Survival rates were monitored over 72 h. All data are shown as the mean ± SD (n = 6). $^{\#}P < 0.05$, significantly different from control group; $^{*}P < 0.05$, significantly different from LPS alone.